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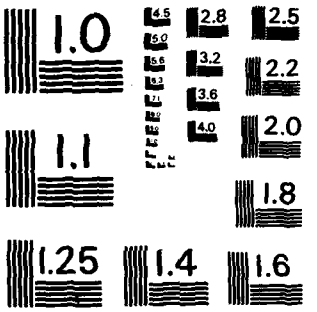

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The research project is concerned with the detection of premalignant and malignant cells induced in vivo in a novel assay system the granuloma pouch assay. Cells exposed to carcinogens in vivo can be studies for DNA damage, chromosomal aberrations, specific locus mutations and cell transformations. Various assays were developed to investigate the growth characteristics of normal, carcinogen-exposed and transformed granuloma pouch cells.

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These included: primary cloning efficiencies under optimal growth conditions, growth in serum deficient and Ca depleted media, growth in soft agar, growth in athymic mice and rats. Cells were also studied after passage through athymic mice.

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CORRELATION OF MUTAGENIC, CARCINOGENIC AND CO-CARCINOGENIC EFFECTS OF CHEMICAL SUBSTANCES (GRANULOMA POUCH ASSAY)

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1. PREFACE

Work described in this report is part of a research project in which new in vivo methods for the detection and characterization of mutagenic and carcinogenic chemicals are developed. A substantial part of this effort is supported by the Swiss Federal Institute of Technology and the University of Zurich. The AFSC grant is used to intensify work aimed at the detection of malignant and pre-malignant cells exposed in vivo to various chemicals.

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4. Abbreviations and Symbols

AP_4A:	Diadenosine tetraphosphate
FCS:	fetal calf serum
GP:	Granuloma pouch
GPA:	Granuloma pouch assay
SCE:	Sister chromatide exchange

5. Work performed in the first year

5.1. General Concepts

The detection of mutagenic and carcinogenic chemicals is based mainly on in vitro test with microorganisms and mammalian cells and on the life-time rodent bioassay respectively. Much new knowledge has accumulated in recent years on the molecular mechanisms of the "genotoxic" chemicals that damage DNA, induce mutations and are often also associated with a higher incidence of tumors in laboratory animals exposed during long periods of time.

Many mechanisms have now been identified which can either enhance or retard the biological reactions that are involved in DNA damage, mutation and tumor development. In addition, salvage pathways have been identified which permit the organism to repair DNA damage and the mutated genome, and which recognize and destroy malignant cells. Many of these salvage mechanisms can again be either enhanced or impaired by chemical substances. Finally, it has been recognized that cells which were transformed by chemical, viral or physical insults very often remain quiescent and are under the control of the surrounding normal cell population. Chemical and other (e.g. hormonal, perhaps even psychic) factors are now known which permit the transformed cells to express their malignant properties and to grow.

Many chemicals that affect the complex processes which lead from the initial DNA lesion to a lethal tumor do not act on the genome. Thus, it is not justified to limit the mutagenicity testing and the search for carcinogens to the detection of genotoxic agents. In order to arrive at a better understanding of the many factors involved in mutagenicity and carcinogenicity, in vivo model systems must be developed, in which certain key steps in the process of tumor development can be assessed.

5.2. The Granuloma Pouch Assay

The animal model in which a variety of mutagenic and carcinogenic processes can be analyzed under in vivo conditions was developed by our research group. It is called granuloma pouch assay, because it studies the chemically induced changes in a granulation tissue which grows at the inside of a subcutaneous air pouch in rats.

This experimental model has many advantages: A well defined cell population consisting of fibroblasts, endothelial cells and macrophages can be induced to grow rapidly by simply injecting air under the rat skin. These cells can now be exposed to chemicals during their most voulnerable period, i.e. while they are dividing rapidly. The chemicals can be given locally where they interact directly with the growing cells, or they can be administered systemically and reach the granuloma tissue either in unchanged or in metabolized form via the blood stream.

It was found that cells harvested from the granuloma pouch grow very well in tissue culture. Thus, they cannot only be analyzed for DNA damage (strand breakage etc), chromosomal aberration and sister chromatide exchange (SCE), but also for the presence of specific locus mutations through the technique of growth in selective media.

Finally, tumors developing in the area of the granuloma pouch can be readily detected by palpation.
Several papers describe the granuloma pouch assay. They are listed under "Bibliography" at the end of this report.

5.3. Growth Characteristics of Granuloma Cells

Three cell populations can be obtained from the granuloma pouch and can be studied individually, namely:

a) normal granuloma cells and various subfractions separated by elutriation

b) "carcinogen-exposed" granuloma cells, e.g. cells that were treated once or repeatedly, locally or systemically, with a genotoxic carcinogen. This population contains many mutated and damaged cells, but also pre-malignant cells. By sampling this population at various time points, the effects of time on the fixation of the genetic damage and the ability to express certain growth characteristics can be studied.

c) malignant cells. These are fibrosarcoma cells of a great variety of histological types which develop in the granuloma pouch, if the cells had a single or repeated contact with a carcinogen.

The following growth characteristics were investigated with normal and malignant cells (studies with "carcinogen-exposed" cells will follow when all assay systems are standardized).

a) growth under optimal culture conditions
b) growth under the influence of biological response modifiers
c) growth in serum-depleted medium
d) growth in calcium depleted medium
e) growth in soft agar
f) growth in living hosts.

a) Growth under optimal culture conditions

The end point to measure growth was the stimulation of individual cells to form clones of more than 20 cells within 6 days. In order to do this, the pH and pO2 were adapted to those found under in vivo conditions (1). In a broad evaluation with three different media including standardized serums we found that the standardized Dulbecco's modified minimal essential medium with a pO2 of 30-40 mm Hg and 10 % FCS was optimal for growth. Moreover, recovery of specific locus mutations from carcinogen-exposed cells was enhanced by the optimized culture conditions. The slightly acid pH found in our measurements in tumor tissue did not clearly improve culture conditions.

These optimal growth conditions however were not specific for transformed cells. Untreated GP-cells showed also an enhanced cloning efficiency under low pO2.

b) Growth under the influence of biological response modifiers

These studies were conducted with malignant granuloma pouch cells. It was found that Al $(OH)_3$, Vitamin E and AP_4A (Diadenosine tetraphosphate) did not affect cloning efficiency. They will not be tested further. Two other chemicals were selected as candidates for growth stimulation, i.e. 1-isoamyl-3-isobutyl xanthine (MIX) and adenosine.

(1) Maier, P., Weibel, B. and Zbinden, G. Influence of pO2 on primary cultures of cells derived from granulation tissue of rats. Experientia 38, 744, 1982.

c) Growth in serum depleted medium

It is suggested that in vivo or in vitro transformed cells are deficient in growth regulation at a hypothetical restriction point (R-point). A method to investigate this phenomenon is to determine the arresting point. This was done with in vivo transformed cells after culture in 0.3 % serum using a cytofluorometric staining method developed at our institute (1, 2). This investigation was completed, and we found that only 13 out of the 24 cultured populations (54 %) from fibrosarcomas grow in serum depleted medium. The arresting point for the remaining cell populations was in the G_0 phase, as it was also found in normal cells isolated from GP tissue.

d) Growth in calcium depleted medium

It is known that calcium and cyclic AMP are involved in the control of cell proliferation (3). Various epithelial and mesenchymal tumor cells initiate DNA synthesis, multiply and form colonies in low calcium media that do not permit the proliferation of their nontumorigenic counterparts.

A medium was developed which differs only in the low calcium (0.02 mM Ca^{2+}) from the standard Dulbeccos modified Eagles medium (1.8 mM Ca^{2+}). The neoplastic index was defined as cloning efficiency of subcultured, in vivo transformed cells in low Ca^{2+}/ Cloning efficiency of cells in standard medium.

(1) Moser, G., Maier, P. and Zbinden, G. Go-G1 transition of rat hepatocytes detected by changes in nuclear chromatin condensation after in vivo treatment with phenobarbital. Cancer Letters 19, 253-261, 1983.

(2) Moser, G., Maier, P. and Zbinden, G. Determination of cell cycle position in cells from whole animals. Experientia in press.

(3) Swierenga, S.H.H. Regulation of poliferation of normal and neoplastic rat liver cells by calcium and cyclic AMP. Ann. NY Acad. Sci. Vol. 349 294-311, 1980.

For untreated GP-cells, under physiological pO2, this neoplastic index was determined as $0.083^{+}0.046$ (n=5). With one exception (21/III) all isolated in vivo transformed cells tested showed significant growth in Ca^{2+}-depleted medium. This endpoint has so far a predictive value of 89 % and is therefore a valuable criterion to culture selectively neoplastic cells and hopefully also preneoplastic cells.

e) Growth in soft agar

Most likely the microenvironment produced by individual tumor cells in soft agar is growth promoting for themselves. From freshly isolated primary cell populations of GP-derived fibrosarcomas, 20 out of the 24 tested (83 %) formed clones in soft agar and none of the untreated GP-cells. The cloning efficiency from growing cells varies between 0.1 and 4 %. This endpoint has therefore a high predictive value for the detection of preneoplastic cells.

f) Growth in living hosts

Species and strain

With the support of a breeding facility in Switzerland, we evaluated various nude mouse and nude rat strains as hosts for in vivo transformed cells. Tumor formation in these athymic mice and rats is considered to be the ultimate proof of malignancy of implanted cells. The following five strains were evaluated: Mice: C57 b nu/nu, Fü Alb nu/nu, Balb c nu/nu, rats: Fi 344 rnu/rnu, DA rnu/rnu.

In pilot experiments we demonstrated that the observation time for tumor growth can be restricted to a period of 3 months.
The lag time (the time until subcutaneous nodules of at least 3 mm in diameter are detectable) was investigated in the two species rat and mouse. (Table 1) For example, with one representative tumor cell population (11/0), the lag period varied between 3 - 14 days in the three mouse strains and between 12 - 15 days in the two rat strains. There was no consistent pattern with regard to susceptibility of the various strains.

Space required for animal housing favors the use of mice as hosts. The health of the Fü Alb nu/nu was best among the three strains available. Further studies in living hosts will be restricted to this strain, available through a reputed breeding facility in Switzerland.

Number of cells injected

In a serie of experiments with 14 tumor cell populations, the relationship between lag time and number of injected cells was evaluated. 10^6-10^5 transformed cells injected subcutaneously induced local fibrosarcomas. The lag time was correlated with the number of cells inoculated and varied in individual tumor cell populations between 6 and 35 days.

Influence of normal cells

After isolation of in vivo treated GP cells, we assume that only a small number of cells will show a preneoplastic phenotype. Their outgrow to tumors in situ or in living hosts could be impaired by normal cells. Therefore the influence of freshly isolated subcultured cells from untreated animals on growth of in vivo transformed cells in a living host was investigated. Compared with the same number of injected transformed cells, we found a shorter lag time when transformed cells were mixed with untreated GP-cells.

This unexpected result was substantiated so far with 6 isolated tumor cell populations. When isolated cells from nu/nu mice were mixed with transformed rat cells, this growth stimulation was not detectable. We conclude that subpopulations of untreated rat cells are able to stimulate growth of their preneoplastic counterpart. Reconstitution and definition of this stimulus in vitro might help to define mechanisms involved during the prolonged lag time observed in carcinogenesis experiments and then can be used for the detection of preneoplastic cells.

Influence of in vitro handling

Growth of freshly isolated preneoplastic cells in vivo might be impaired by the isolation procedures (dissociation with proteases) or by other non avoidable handling procedures in vitro.
From 5 representative tumor cell populations we compared the lag time in vivo of i) freshly isolated cells, ii) of cells stored for several months in liquid nitrogen and iii) of cells cultured in petri dishes or in soft agar. No significant difference was found.

Influence of host passage

From 14 cell populations growing in living host, we investigated whether passage through the host selects for malignant cells. The morphology of secondary subcutaneous tumors and the cloning efficiency of their isolated cells in petri dishes as well as in soft agar were not changed by the host passage.

Comparison of the various assays

With 14 tumor cell lines isolated from malignant GP tissue, the various assays were compared. The results are shown in Table 2. All 14 populations formed fibrosarcomas in nude mice. The histopathological type was always very similar to that found in the original tumor. This indicates that growth in living hosts detects a broad spectrum of neoplastic cells.

The other assays (primary cloning efficiency, growth in soft agar, growth in serum depleted and Ca depleted medium and primary cloning efficiency and growth in soft agar of cells obtained from nude mice) showed marked differences from one tumor cell line to the other.

5.4. Tumor development in GP (1)

The effect of a single dose treatment with procarbazine of GP cells during the time of maximal growth was investigated. Local fibrosarcomas were induced regardless whether the carcinogen was given

(1) Zbinden, G. and Maier, P. Single dose carcinogenicity of procarbazine in rats. Cancer letters in press

locally (into the pouch) or systemically. The administration of croton oil before procarbazine increased the tumor yield. This is the first time that such an effect could be observed. Treatment of animals exposed to a carcinogen with phorbol esters after carcinogen exposure did not significantly accelerate tumor development.

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6. Zbinden, G., Maier, P. and Alder S. Granuloma pouch assay. III. Enhancement of the carcinogenic effect of N-methyl-N'-nitro-N-nitrosoguanidine. Arch. Toxicol. 45, 227-232, 1980.

Table 1

Lag time (days) of in vivo transformed cells in athymic hosts

Tumor	nude rats		nude mice		
	Fi 344	Da	C 57	Balb C	Fü Alb
11/O	12	15	13	3	11
	nt	nt	6	12	9
14/II	11	14	11/11	14/11	11
	nt	10/10	3	nt	nt
19/III	nt	nt	8	9	6
21/O	nt	nt	50	59	43
21/III	ng	77	nt	nt	nt
	nt	35	nt	nt	29/18/16
41/III	20	nt	9	nt	nt
43/III	15/23	nt	14	nt	nt
49/III	ng	20	nt	15	15/24

nt not tested
ng no growth

TABLE 2

Growth characteristics of 14 Tumor Cell Lines isolated from GP.

	GP Cells					Cells isolated from nude mice	
Cell line	I cloning efficiency	growth soft agar	growth serumdepletion	growth Ca depletion	growth nude mice	I cloning efficiency	growth soft agar
10/II	<1	+	+	+	+	<1	+
11/O	<1	+	-	+	+	<1	+
13/O	<1	+	-	+	+	<1	+
13/I	<1	+	-	nt	+	<1	+
14/II	<1	+	+	+	+	nt	+
16/O	1.6	+	nt	nt	+	<1	-
16/I	65	+	+	+	+	nt	+
19/III	5	+	-	+	+	nt	+
21/O	<1	+	-	nt	+	<1	-
21/III	<1	+	+	-	+	nt	+
41/III	64	+	-	nt	+	21	+
42/II	10	+	-	nt	+	5.6	+
43/III	<1	-	+	+	+	± 1	-
49/III	<1	+	-	nt	+	± 1	+
untreated GP cells	75.4	-	-	-	-		-

nt = not tested

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